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Antiviral and Anti-Inflammatory Effects of Rosmarinic Acid in an Experimental Murine Model of Japanese Encephalitis[▽]

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Rosmarinic acid (RA) reduced the mortality of mice infected with Japanese encephalitis virus (JEV). Significant decreases in viral loads ($P < 0.001$) and proinflammatory cytokine levels ($P < 0.001$) were observed in JEV-infected animals treated with RA compared to levels in infected mice without treatment, at 8 to 9 days postinfection.

Flaviviruses are important human pathogens causing a variety of diseases ranging from mild febrile illnesses to severe encephalitis. Among them, Japanese encephalitis virus (JEV) targets the central nervous system and is a major cause of acute encephalopathy in children (1). Clinically, infection with JEV results in increased levels of inflammatory mediators like tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), IL-8, and RANTES in serum and cerebrospinal fluid (3, 9, 12), which bear a direct correlation with the mortality rate for JE patients (14). As increased microglial activation and the subsequent induction of proinflammatory mediators like TNF-α, IL-6, and MCP-1 following JEV infection influence the outcome of viral pathogenesis (2), it is possible that the increased microglial activation also triggers bystander damage, since infected animals eventually succumb to infection.

Rosmarinic acid (RA), a phenolic compound found in various Labiatae herbs (6, 11), possesses several anti-inflammatory properties (7, 10, 16). Besides, the antioxidative property of RA has been demonstrated by its ability to reduce liver injury induced by D-galactosamine (15) and lipopolysaccharides (8), through the scavenging of superoxide molecules (13) and the inhibition of cyclo-oxygenase-2 (Cox-2).

In the present study, we investigated the efficacy of RA as a therapy against murine JE, using four groups of 15 mice each: a control group injected with phosphate-buffered saline (PBS) only, a JEV-infected group, a JEV-infected and RA-treated group, and an RA-treated group. In the JEV-infected group, 4- to 5-week-old BALB/c mice of either sex were infected intravenously (through the tail vein) with a lethal dose of 3×10^5 PFU of JEV (GP78 strain). From day 5 postinfection, the animals started to show symptoms of JEV, including limb paralysis, poor pain response, restriction of movements, piloerection, body stiffening, and whole-body tremor. Within the ninth day postinfection, all animals in the JEV-infected group succumbed to infection. In the JEV-infected and RA-treated group, RA (Tocris Bioscience) was dissolved in 1× PBS at a concentration of 2.0 mg/ml and stored at −20°C. One day following the virus inoculation, the animals started receiving RA intraperitoneally, twice daily (25 mg/kg of body weight) until the first animal died from the group of infected animals which did not receive any RA treatment. All experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee of the National Brain Research Centre (NBRC).

Animals from the control (injected with only PBS), the JEV-infected, and the JEV-infected and RA-treated groups were perfused with PBS containing 7 U/ml heparin and then with a fixative containing 2.5% paraformaldehyde in PBS, and their brains were processed for cryostat sectioning. The sections were stained with Iba-1 (Wako Chemical, Japan), a marker for activated microglia (4). Activated microglia were counted from five different fields of the cortex by using the software IM50 (Leica), and images were captured under ×20 magnification (2). The average numbers of activated microglia were plotted as a graph.

Western blot analysis was performed with protein isolated from brain tissues from all four groups of animals at 8 to 9 days postinfection (depending upon the mortality of the infected animals) (2). Briefly, 10 μg of each sample was electrophoresed and transferred onto a nitrocellulose membrane. The membranes were then blocked and probed with several primary antibodies, including the JEV Nakayama strain and Cox-2 antibodies (1:1,000 dilution; Chemicon), phospho-NF-κB (pNF-κB) and IκB-α (1:1,000 dilution; Cell Signaling Technology), and β-tubulin (1:1,000 dilution; Santa Cruz Biotechnology). Appropriate horseradish peroxidase-conjugated secondary antibodies were used for all samples. Chemiluminescence reagent blots were developed, and images were captured and analyzed using the Chemigenius bioimaging system (Syngene). We have also performed Western blot analysis with protein isolated from JEV-infected and RA-treated animals at death.

The mouse cytokine bead array (CBA) kit (BD Biosciences) was used to quantitatively measure cytokine levels in the brain tissue lysates isolated from all four groups of animals, and analysis was performed as described previously (2). The CBA was also performed with protein isolated from the group of JEV-infected and RA-treated animals at death. BV-2, a mouse microglial cell line, was either mock infected or adsorbed with JEV (multiplicity of infection, 5) for 1 h. After adsorption, unbound viruses were removed by washing the cells with PBS,

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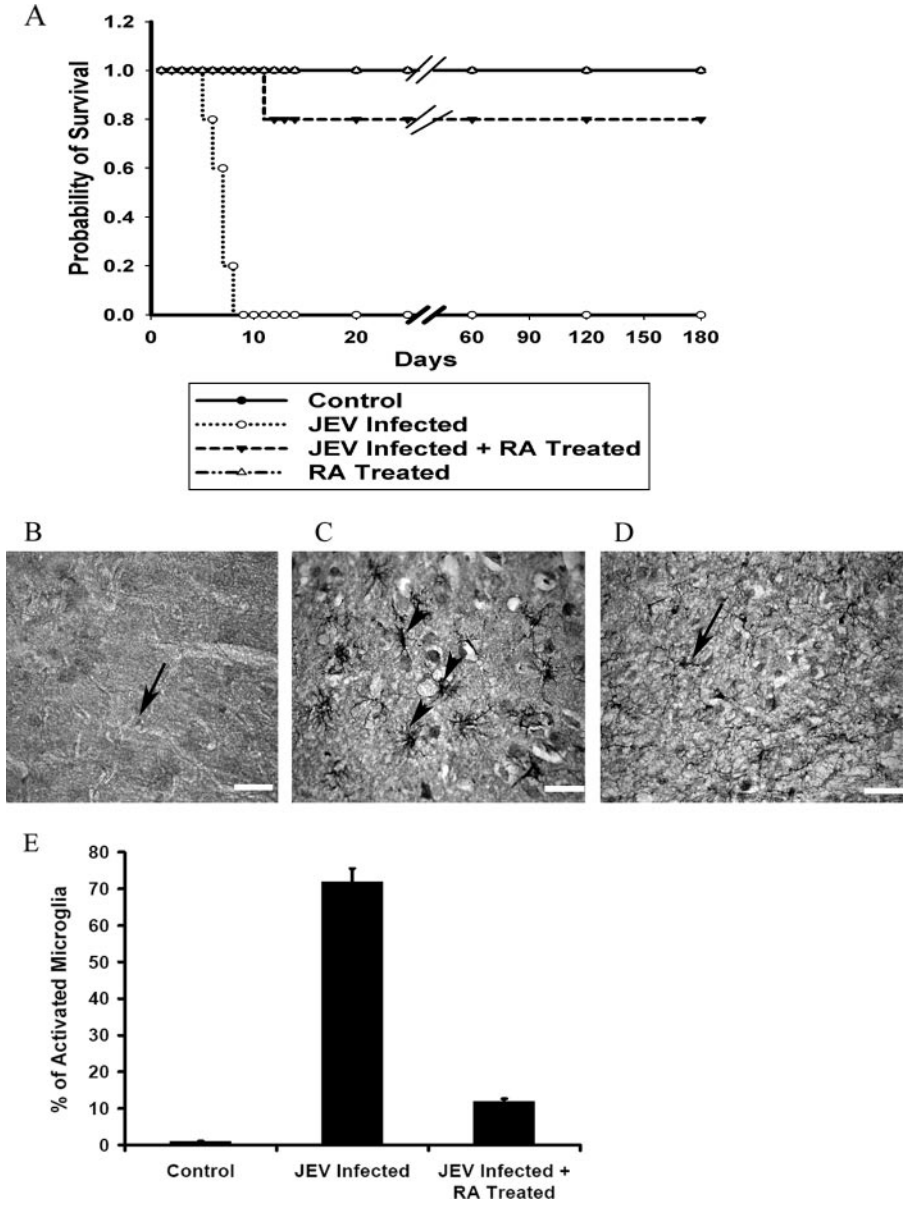

FIG. 1. RA treatment significantly increases the survival of JEV-infected mice. (A) Survival of mice infected with 3×10^5 PFU of JEV was significantly increased in groups that received RA treatment (15 mice for each group). Treatment with RA alone has no significant effect on survival. Observation of animal survival experiments was performed in a masked manner to avoid bias toward any one group of animals. (B to E) Cryostat sections from control (B), JEV-infected (C), and JEV-infected and RA-treated (D) mouse brains were processed for Iba-1. While the control sections exhibited only resting microglia (B; arrow), the infected brains showed the presence of activated microglia (C; arrowheads). The group of JEV-infected and RA-treated mice had mostly resting microglia (D; arrow). Scale bar, 25 μm. (E) Iba-1-positive activated microglia were counted and plotted as a graph. Values represent the means ± standard errors of the means (SEM) from five random fields in three animals in each group ($P < 0.001$).

and the cells were incubated in fresh serum-free medium either in the presence or absence of 25 μM RA for an additional 18 h. Following incubation, the cell lysate was collected and the CBA was performed. Western blot analysis and the CBA were also performed on the proteins isolated from the JEV-infected animals that succumbed, even after RA treatment.

All comparisons between groups were performed using one-way analysis of variance, with the Bonferroni method used for post hoc pairwise multiple comparisons to detect P values of <0.05 between individual group means.

RA treatment following JEV infection reduced the mortality rate to 20% (12 out of 15 animals survived following RA treatment of the JEV-infected and RA-treated group) (Fig. 1A). While all infected animals that did not receive any RA treatment succumbed to infection, treatment with RA alone had no effect on the behavioral outcome of the animals (data not shown). Immunohistochemistry revealed both qualitative and quantitative differences in microglial activation in the infected animals treated with RA compared to the animals infected without treatment. In the brains of the JEV-infected

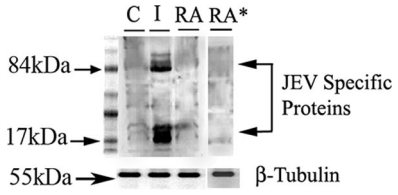

FIG. 2. Antiviral efficacy of RA. C, control mice; I, JEV-infected mice; RA, JEV-infected and RA-treated mice; RA*, JEV-infected and RA-treated mice (dead). RA treatment in JEV-infected mice completely reduced the levels of viral proteins. Proteins isolated from control, JEV-infected, and JEV-infected and RA-treated BALB/c mice were analyzed by immunoblot analysis. A significant decrease in the levels of JEV-specific proteins (84 kDa and 17 kDa) was observed in RA-treated samples compared to levels in infected mice. Data shown are representative of four individual animals from each group.

group, star-shaped "activated" microglia (Fig. 1C) appeared more frequently (by more than 30-fold) than in the brains of the control group (Fig. 1B) or of the JEV-infected and RA-treated group (Fig. 1D). A significant induction of JEV-specific proteins (17 kDa and 84 kDa) was observed in the infected group (Fig. 2). RA treatment completely abolished the expression of viral proteins and significantly reduced viral mRNA transcripts (data not shown). Interestingly, viral proteins were also absent in the JEV-infected mice which succumbed even after RA treatment.

As shown in Fig. 3A, RA dramatically reduced the levels of proinflammatory cytokines and a chemokine. Significant ($P < 0.001$) 5-, 18-, 6-, 100-, and 9-fold decreases in the levels of IL-12, TNF-α, gamma interferon (IFN-γ), MCP-1, and IL-6, respectively, were observed in the infected animals treated with RA compared to the levels in infected animals without treatment. In contrast, infected mice that succumbed even after RA treatment had significantly high levels of proinflammatory cytokines compared to levels in uninfected mice (Fig. 3A, right panel). In line with studies reporting that RA acts as a downstream inhibitor of IK kinase-β activity (5), Western blot analysis revealed a significant ($P < 0.05$) increase in IκB-α levels and considerable decreases in both the pNF-κB and Cox-2 levels in infected animals treated with RA compared to levels in animals that were infected but not treated (Fig. 3B). Interestingly, the infected animals that died even after RA treatment had increased levels of pNF-κB and Cox-2 and decreased levels of IκB-α compared to the control group. High levels of proinflammatory mediators in infected animals that succumbed even after RA treatment, despite decreased viral

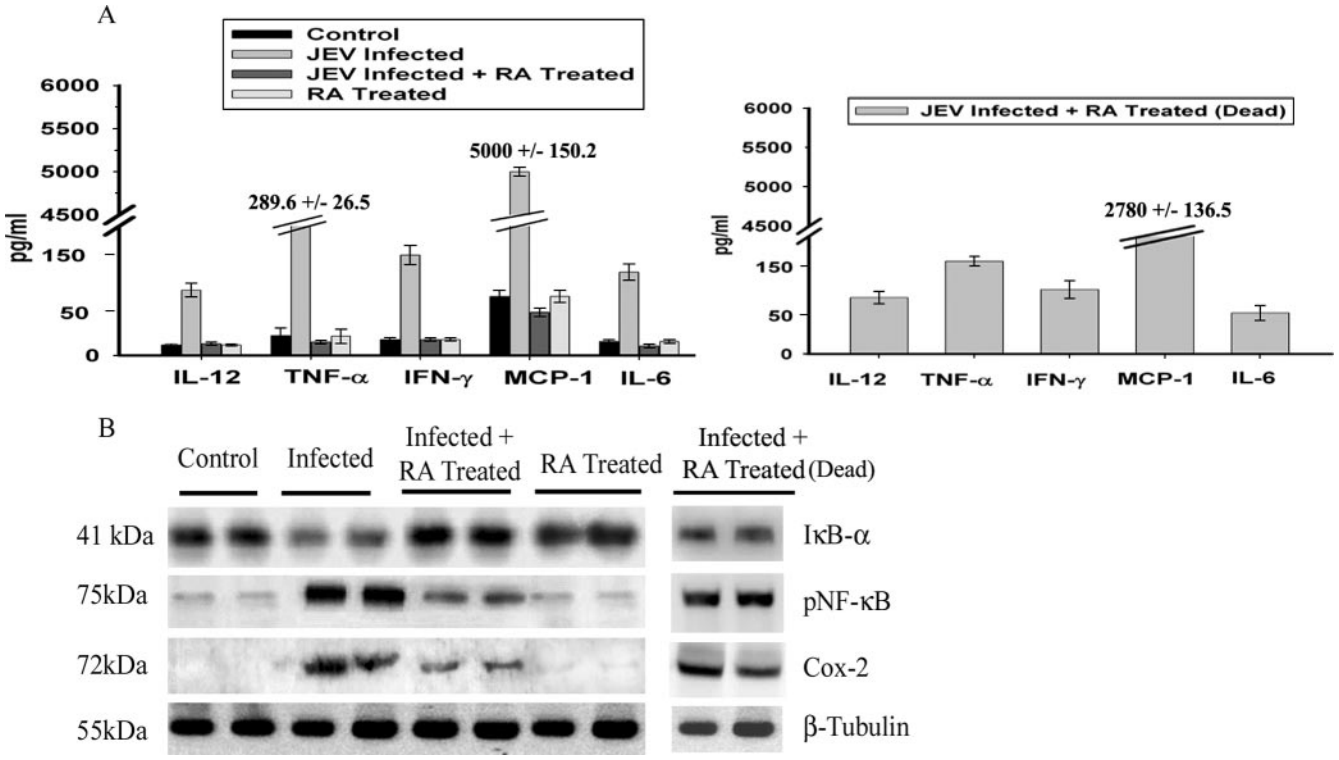

FIG. 3. RA abrogates the increased expression of proinflammatory mediators. (A) Expression of IL-12, TNF-α, IFN-γ, MCP-1, and IL-6 was observed by CBA in control animals, JEV-infected animals, JEV-infected and RA-treated animals, animals treated with RA alone, and JEV-infected and RA-treated animals at death. Levels of IL-12, TNF-α, IFN-γ, MCP-1, and IL-6 were significantly reduced in RA-treated samples compared to levels in infected mice. $P < 0.001$ (mean ± SEM). JEV-infected mice that succumbed even after RA treatment had significantly high levels of proinflammatory cytokines compared to uninfected mice. $P < 0.001$ (mean ± SEM) (four mice for each group). (B) Protein levels of control, JEV-infected, JEV-infected and RA-treated, RA-treated, and JEV-infected and RA-treated animals at death were analyzed by immunoblotting. Significant reductions in the levels of pNF-κB and Cox-2 in RA-treated samples were observed compared to levels in infected samples ($P < 0.05$). RA treatment reversed the level of IκB-α ($P < 0.05$). Mice that died even after RA treatment had increased levels of pNF-κB and Cox-2 and decreased levels of IκB-α compared to the control group. Data shown are for two individual animals from a total of four animals in each group.

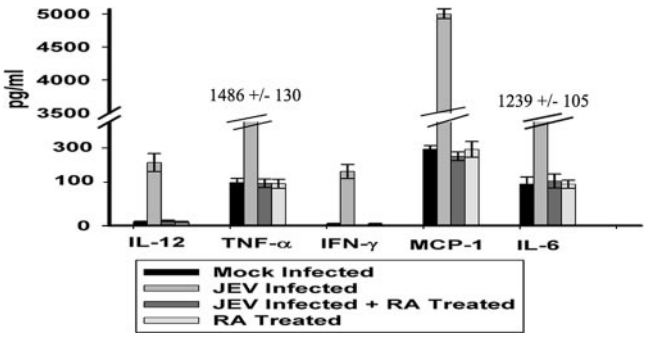

FIG. 4. RA decreases the proinflammatory mediators in vitro. Mouse microglial cell line BV-2 was used to study the in vitro induction of proinflammatory cytokines and a chemokine following JEV infection. There were significant reductions in the levels of IL-6, MCP-1, IL-12, IFN-γ, and TNF-α in JEV-infected and RA-treated samples compared to levels in JEV-infected samples. Values represent the means ± SEM from three independent experiments performed in duplicate ($P < 0.05$).

loads, suggest that besides reducing viral loads, proinflammatory mediator levels are important in determining the final outcome of the disease.

To confirm the in vivo findings that RA modulates the release of proinflammatory cytokines and chemokines following JE, we determined the levels of proinflammatory mediators in the mouse microglial cell line BV-2 infected with JEV in vitro. While JEV infection of the BV-2 cells increased the release of various proinflammatory cytokines, treatment of the infected BV-2 cells with RA significantly ($P < 0.001$) reduced the levels of IL-12, TNF-α, IFN-γ, MCP-1, and IL-6 by 13-, 14-, 12-, 25-, and 12.5-fold, respectively, compared to levels in the RA-untreated infected animals (Fig. 4). No significant changes with RA treatment alone were observed.

In conclusion, our studies suggest that RA acts as a potent antiviral agent against JE. Results from our in vivo experiments clearly indicate that RA reduces the (i) viral replication within the brain and (ii) secondary inflammation resulting from microglial activation, thereby suggesting its potential for treating JE. Both the antiviral and anti-inflammatory effects of RA are essential for reducing the severity of diseases induced by JEV. The studies presented here recommend RA as a strong candidate for further consideration as a therapeutic measure to reduce the neurological complications observed in JE patients.

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